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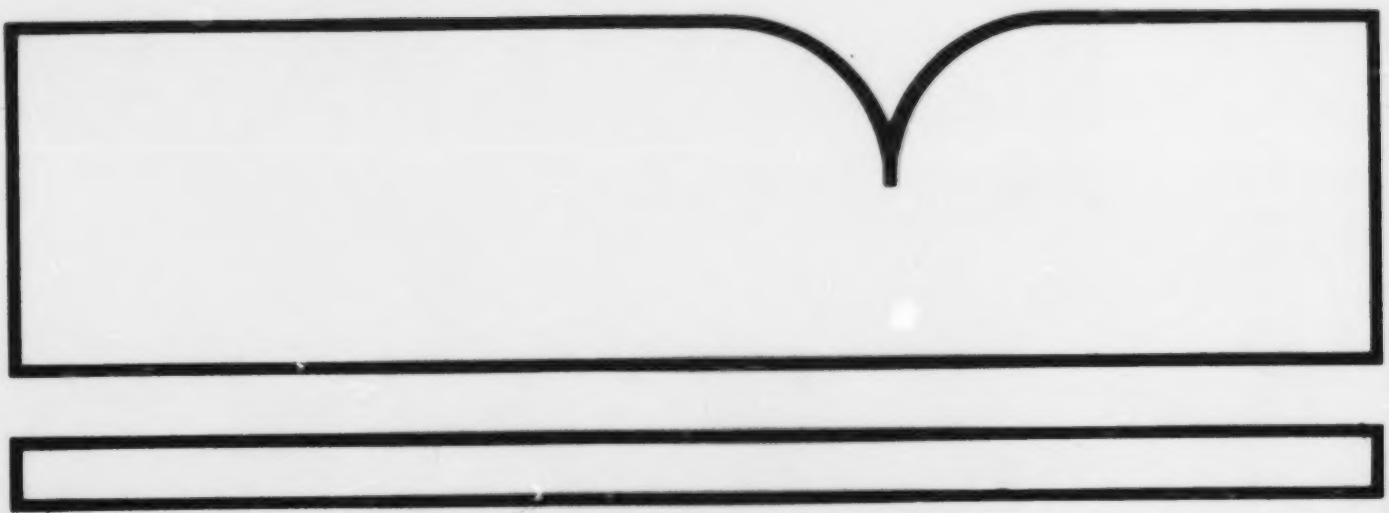

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GROWTH OF *LEGIONELLA PNEUMOPHILA* IN CONTINUOUS CULTURE AND ITS SENSITIVITY TO INACTIVATION BY CHLORINE DIOXIDE

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16. ABSTRACT

A complex medium, that permitted growth of Legionella pneumophila under chemostat conditions, was developed. The elimination of iron (III) was necessary to prevent catalytic oxidation of cysteine present in the medium. The inactivation of Legionella pneumophila populations grown under various nutrient conditions was studied. The results indicated that populations grown under low nutrient conditions were more resistant to inactivation than population grown under high nutrient conditions.

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Substantial differences in sensitivity of bacteria to inactivation by disinfectants have been attributed to the conditions experienced by the organisms immediately prior to treatment (2,5,). The physiology, growth rate, and gross morphology of bacteria and the ensuing responses to environmental stresses (e.g., disinfectants) can be altered significantly by growth parameters such as nutrient concentration and composition, oxygen tension, and temperature (9,10). These parameters cannot be adequately controlled with conventional batch culture techniques, in which the environmental conditions change drastically during growth yielding populations of unknown phenotype. These problems are confounded because laboratory cultures which can be more sensitive by orders of magnitude than naturally occurring populations (4) have usually been used in disinfection studies.

Several Legionella species isolated from a diverse set of aquatic environments (8), have been reported to exhibit differing sensitivities to disinfectants (6,15). Also, there is some controversy concerning the growth requirements of Legionella, especially with respect to L-cysteine, iron (III), and oxygen (7,11,12,14). Therefore, the objectives of this study were to utilize the continuous culture approach to develop additional information on the growth requirements of Legionella, and evaluate the effects of various growth conditions on the sensitivity of the cultures to disinfectants.

The medium of Ristroph et al. (12) was modified for use in the chemostat. The modified medium contains, per liter: yeast extract (Difco), 10g; L-cysteine, 0.2g; and starch, 0.25g. The pH is adjusted to 6.9 using 1N KOH. Lastly, the medium was filter-sterilized through a Seitz S3 filter using nitrogen. Nitrogen was bubbled continuously through the 10 liter reservoir to maintain a reducing environment. It was necessary to remove iron (III) from the medium to minimize the catalytic oxidation of cysteine that can occur

under forced aeration. Using the methods of Roberts and Rouser (13) and Alexander (1) cysteine at a concentration of 0.4 gl^{-1} in the presence of iron (III) was undetectable after eleven minutes. Based on the reaction stoichiometry of Taylor et al. (16) in the reaction of cysteine and oxygen (4:1) the cysteine concentration was estimated to approach zero in less than 60 minutes under typical continuous culture conditions in the presence of iron (III). The removal of iron (iii) minimized the problem of cysteine oxidation. Moreover, the apparent increased stability of the L-cysteine in this medium permittd a yield approximately 3.5 times greater than could be achieved in batch cultur using the conventional yeast extract medium supplemented with L-cysteine and iron (III) as ferric pyrophosphate. The addition of the starch-enhanced growth, presumably bcause of its role as an absorbant of toxic substances that may be produced at the surface of the immersion heater in the chemostat vessel. Ristroph et al. (12) suggested that fatty acids which inhibit Legionella can be produced during heating of yeast extract.

The chemostat inoculum was obtained by streaking a stock suspension, previously stored at -70°C, onto a Charcoal Yeast Extract (CYE) agar slant which was then incubated for 48-72 h at 37°C. The growth on the slant was then washed into a 250ml flask containing 100 ml of chemostat medium (previously described) and incubated in a shaking waterbath at 37°C for 24 h. The liquid culture was transferred into the 2 liter chemostat vessel which was purged with nitrogen. The culture was then diluted with medium to an optical density at 660nm (OD_{660}) of >0.15. Once an OD_{660} of about 0.40 was achieved (after 4-6 days), positive aeration and nutrient pumping was started. Steady-state cultures can be harvested in two to five days depending on the dilution rate (D, h^{-1}).

L. pneumophila was grown successfully under a variety of growth conditins using batch a..d continuous culture. Two dilution rates (D = 0.06 h^{-1} and D = 0.15 h^{-1}) and two temperatures (37° amd 44°C) were used. D = 0.06 h^{-1} represents the slow growth rate and D = 0.15 h^{-1} reprsents an intermediate rate. The 37°C temperature was the optimum, whereas 44°C represents a more harsh environment throught to exist in thermal effluents and hot tap water systems. Aeration utilized ambient air or a mixture of 95% air and 5% CO_2. The flow rates of each gas have ranged from 20 to 150 l min^{-1} of either gas, yield ing l to 38% saturatin of dissolved oxygen.

Steady-state OD values ranged from 0.64 to 1.20, depending on the conditions employed. High catalase activity was observed under all of the aforementined growth conditions. Pigment production observed in the stationary phase batch culturesi the iron-containing media, was not noted under chemostat conditions. A requirements for a titrant to maintain the ph at 6.9 was dependent on the form of aeration. Air (95%) plus CO_2 (5%) required the addition of base (0.5 N KOH), whereas, without CO_2 (5%), acid (0.5N HCl) was required (e.g., at D = 0.06 h^{-1}), 37°C, 2 mM acid h^{-1} was consumed).

The sensitivity of the chemostat populations to chlorine dioxide (ClO_2) was investigated in triplicate experiments using populations grown under three different conditions. The results of the inactivation experiments are shown in Figure 1. Legionella cultures at a particular steady-state were harvested and centrifuged, then resuspended in phosphate buffer at pH 7 to yield 10^7 CFU ml^{-1}. A dose of 0.75 mg ClO_2/liter was used in each experiment. The experiments were done in triplicate. The more slowly grown cells (D = 0.06 h^{-1}) from the chemostat were about two orders of magnitude more resistant than either of the batch grown populations. These data are analogous to the data obtained in previous experiments using E. coli (3). The two batch-grown

populations differed with respect to the presence of iron (III) in the medium and the concentration of L-cysteine and exhibited no significant differences in sensitivity. Inactivation experiments using cultures grown under other conditions are in progress.

When comparing these sensitivity data with those obtained for E. coli (3), the Legionella in all three cases were more resistant in two respects. First, the initial inactivation rate for Legionella was much more slower than that for E. coli. Second, the extent of kill (t = 15 min) was much less for Legionella.

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Figure 1. Effect of growth environment on sensitivity of L. pneumophila to a dose of 0.75 mg ClO_2 l^{-1}. (O) chemostat cultures grown at D = 0.06 h^{-1}, 37°C, using air at 20ℓ min^{-1}; (Δ) batch cultures, 37°C on a shaker, harvested at 36 h; (□) batch cultures, 37°C on a shaker, harvested at 24 h. Medium for (□) was different from the other two in that it contained ferric pyrophosphate (0.25gl^{-1}) and additional L-cysteine (0.4gl^{-1}).

FIGURE 1.